Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Delvaeye M, Noris M, De Vriese A, et al. Thrombomodulin mutations in atypical hemolytic–uremic syndrome. N Engl J Med 2009;361:345-57.

Supplementary Methods

Patients and controls

All patients with aHUS with available stored DNA, and who had been registered consecutively with the International Registry of Recurrent and Familial HUS/TTP were recruited into this study. The controls were not individually matched. We used two different cohorts of controls. The first cohort comprised 150 healthy controls from the International Registry of Recurrent and Familial HUS/TTP[27]. They were selected for having a median age and female/male ratio and geographic origin (125 from Europe; 20 from North America, white; 5 from Africa) comparable to patients. The geographic origin of patients was as follows: 58% from Italy, 21% from other European countries, 15% from North America, 2% from South America, 2% from Africa, and 1.3% from Middle East countries. To further confirm that the first cohort was representative of a wider population, we studied an additional 230 available DNAs from a second cohort of healthy white caucasians from Europe, relatives of patients with a range of diseases, with a median age older than the first cohort, who had voluntarily donated blood into a databank for research purposes.

aHUS was diagnosed in patients who had one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia (hematocrit <30%, hemoglobin <10 g/dl, serum lactate dehydrogenase >460 U/L, undetectable haptoglobin, fragmented erythrocytes in the blood smear, and platelet count <150,000/μl), associated with acute renal failure. The latter was defined according to the RIFLE classification[28], as an increase in serum creatinine x 3.0 or a decrease in glomerular filtration rate by 75%, or serum creatinine levels ≥ 4 mg/dL with an acute increase of more than 0.5 mg/dL; urine output < 0.3 ml/kg/h for 24 hours or anuria for 12 hours.

Patients with Stx-HUS (defined by the presence of free fecal Stx and/or by positive colony sweeps for Stx production in MacConkey agar culture of stools, by Vero cell assay, and/or serum antibodies measured by ELISA to Stx and/or to lipopolysaccharide of the five major Stx-producing serogroups, O157, O26, O103, O111 and O154[58]) were excluded.

Of the 152 patients, 18 had a familial history of the disease, of whom 3 were from one pedigree and 2 from another one. The others were unrelated, including F635 and F163. For the latter two patients in whom thrombomodulin mutations were found, all available relatives were thereafter recruited and screened. All the other patients had the sporadic form of aHUS and were all unrelated. The 380 controls were unrelated to each other and unrelated to patients.

DNA sequencing and genotypying of SNPs

Genomic DNA was extracted from peripheral blood leukocytes. The entire coding sequence of *THBD*, including the 5' and 3' untranslated regions and approximately 50 bp of 5' and 3' flanking DNA, was directly sequenced on both strands, using 5 pairs of oligonucleotide primers. Sequences that indicated the presence of SNPs were visually confirmed on both strands, and repeated with fresh DNA samples. Further confirmation of SNPs was achieved using the Sequenom MALDI TOF MassArray system[31]. Thus, for SNP analysis, genotyping assays (primers for PCR amplification and the extension probe) were designed using the Sequenom MassARRAY Assay

Design 3.0 software, applying default parameters. Multiplexed PCR was performed with 0.2 units of Taq polymerase and 20 ng of genomic DNA. Thermocycling was at 95°C for 15 min followed by 45 cycles of 95°C for 20s, 56°C for 30s and 72°C for 1 min. Unincorporated dNTPs were deactivated using shrimp alkaline phosphatase, and primer extension was carried out using extension primers, ddNTPs and Thermosequenase DNA polymerase. Reactions were cycled at 94°C for 2 min, followed by 44 cycles of 94°C for 5s, 52°C for 5s and 80°C for 5s. After the addition of a cation exchange resin to remove residual salt from the reactions, the purified primer extension reaction was loaded onto a matrix pad (3-hydroxypicoloinic acid) of a SpectroCHIP (Sequenom) and analyzed using a matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometer.

DNA preparation and cell transfections

Genomic DNA representing the entire coding region of human thrombomodulin was generated by PCR and subcloned into the *EcoR1* sites of the expression vector pcDNA3.1 (Invitrogen). Mutations were introduced with the Quikchange XL site-directed mutagenesis kit (Stratagene, Amsterdam, the Netherlands). Transfections into cultured mammalian cells were performed with Fugene HD (Roche, Brussels, Belgium) in 24-well plates with 0.2 ug DNA per well. Selection was achieved with neomycin (G418, Roche, Belgium) and cells were maintained in DMEM/F12 (CHO-K1) or DMEM (HEK293), supplemented with 10% fetal calf serum (Invitrogen, Merelbeke, Belgium).

Proteins and antibodies

SDS-PAGE and Western immunoblotting was performed by standard techniques. Complement reagents were from Complement Technology, Inc. (Tyler, Texas, USA). Purified proteins were biotinylated with 50x molar excess D-Biotinoyl-e-amidocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim, Brussels, Belgium) in PBS and dialyzed to remove free biotin. Murine monoclonal antibodies (CTM1009) were generated against chondroitin sulfate free human extramembranous thrombomodulin (RSV TM)[59] by standard procedures, purified using Med Hypercel (VWR, USA). The following primary antibodies were used: goat anti-human C3, and goat anti-human CFH. Sheep anti-human C4bBP was from Abcam (Cambridge, UK).

Co-precipitation assays

Proteins that interact with recombinant thrombomodulin (RSV TM[59]), were co-precipitated with sepharose-bound human diisopropylfluorophosphate (DFP)-inactivated thrombin (Enzyme Research Labs, IN) after incubation with RSVTM.

Detection of C3b proteolytic fragments

Recombinant thrombomodulin was incubated with different combinations of C3b 0.5 μg, CFH 100 ng, C4bBP 100 ng, and CFI 100 ng in a volume of 150 ul of PBS at 37^{o}C for 90'. After reactions and separation by SDS-PAGE, C3b proteolytic fragments were analysed by Western blot and quantified by densitometry. The same experiments were performed on the surface of HEK293 cells transfected with pcDNA3.1 (empty vector) or vector expressing thrombomodulin (full-length human thrombomodulin). Equal expression of thrombomodulin in transfected cells was confirmed by Western blot and by ELISA.

Thrombin-mediated TAFI activation

HEK293 cells were incubated at 37^{o}C for 20 mins with 3 ug of TAFI in 150 ul of PBS with 1 mM $CaCl_2$ and 4 U/ml of thrombin. Reactions were stopped with hirudin and the proteins were separated by SDS-PAGE followed by Western immunoblotting to detect TAFIa.

Complement binding assays

Binding of complement proteins to immobilized thrombomodulin was performed with a 2-step procedure. The concentration of thrombomodulin in cell lysates was first quantified using a commercial sandwich ELISA kit (Abcam, Cambridge, UK), but replacing the second anti-thrombomodulin antibody with biotinylated CTM1009.   Lysates with 10 ug/ml thrombomodulin were then co-incubated with 3 ug/ml biotinylated C3b or CFH for exactly 3 hr at room temperature in the wells of the commercial thrombomodulin ELISA plates, which are precoated with anti-thrombomodulin antibodies. After washes, specific binding was quantified with a streptavidin-secondary antibody diluted in the strep-HRP diluent and TNB substrate. Reactions were stopped with H_2SO_4. Absorbance was measured at 495 nm with an ELISA plate reader (Beun De Ronde, Serlabo, Belgium). Non-specific signal, determined from parallel experiments with lysates of cells transfected with empty vector (no thrombomodulin), was subtracted from the result. In all cases, co-incubation of biotinylated protein with a molar excess of the corresponding non-biotinylated protein abrogated the specific signal (not shown). Assays were performed three times in triplicate.

Initiation of complement activation and flow cytometry

Initiation of the complement pathway on CHO-K1 cells was performed as described[32, 33]. CHO-K1 cells stably expressing equal amounts of cell-surface wild-type or mutant forms of thrombomodulin were trypsinized with 0.05% trypsin in PBS, washed in 1% FCS/PBS, and sensitized with anti–CHO antibodies (Cygnus, Southpark, NC, USA) for 30' at 4°C. After washing in gelatin veronal buffer (GVB++) (Complement Technologies Inc., Tyler, Texas, USA), cells were incubated at 37°C for 90' in 10% C6-deficient serum (which allows complement activation to proceed without inducing cell lysis) diluted in GVB ++ buffer, washed twice with 1% FCS/PBS, and incubated at 4^{o}C for 30 minutes with 20 ug/ml of murine monoclonal anti-thrombomodulin antibodies (CTM1009), FITC-conjugated murine anti-human C3c antibodies (11.3 ug/ml, Dakocytomation, Heverlee, Belgium, Heverlee) (identifies C3b and iC3b), or murine anti-human iC3b antibodies (10 ug/ml, Quidel, Osteomedical, the Netherlands, Nijkerk). Species and isotype matched immunoglobulins were used as negative controls. After incubation with an appropriate secondary antibody, cells were washed with 1% FCS/PBS, and fixed with 0.5% paraformaldehyde prior to analysis with the FACScalibur (BD Biosciences, Erembodegem, Belgium). The fold-change in % inactivation of C3b (mean fluorescence intensity of iC3b/C3c) was calculated for wild-type and mutant thrombomodulin-expressing cells, relative to that in non-thrombomodulin expressing cells (i.e. empty vector). Reported results reflect the mean $\pm$ standard error of the mean (SEM) of 3 independent experiments.

Statistical analyses

Data analyses were performed with Graphpad Prism 4 (San Diego, CA, USA). Results are presented as mean ± standard error of the mean (SEM). An unpaired t-test was used to identify differences between groups. Allele frequencies of *THBD* gene variants in patients and controls were compared by the chi-square test. All reported P-values are 2-sided and not adjusted for multiple testing.

Author statements

EM Conway, M Delvaeye, M Noris, G Remuzzi, D Lambrechts, CT Esmon and NL Esmon designed the studies and contributed to all versions of the manuscript. EM Conway and M Noris wrote the major portion of the first draft. M Delvaeye, B Claes, J Del-Favero, S Plaisance and C Zoja performed experiments, gathered and analyzed data. EM Conway, M Noris and D Lambrechts validated data. All authors agreed with submission of the manuscript.

SNP	Location relative to ATG start site of *THBD*	Genotypes and Alleles	Controls n=648 Number (%)	HUS n=152 Number (%)	p-value
rs1040585	9.8 kb 5'	AA	5 (1)	0 (0)	0.23
		AC	62 (11)	9 (7)	
		CC	483 (88)	115 (93)	
		A	72 (7)	9 (4)	0.08
		C	1028 (93)	239 (96)	
rs2424505	5.75 kb 5'	AA	9 (2)	0 (0)	0.33
		AG	94 (16)	19 (15)	
		GG	479 (82)	109 (85)	
		A	112 (10)	19 (7)	0.27
		G	1052 (90)	237 (93)	
rs6076016	4.3 kb 5'	AA	66 (11)	11 (8)	0.21
		AT	265 (45)	56 (40)	
		TT	261 (44)	72 (52)	
		A	397 (34)	78 (28)	0.08
		T	787 (66)	200 (72)	
rs1042580	2.7 kb 3' (in 3'UTR)	AA	217 (36)	52 (34)	0.6
		AG	285 (47)	78 (52)	
		GG	99 (16)	21 (14)	
		A	719 (60)	182 (60)	0.89
		G	483 (40)	120 (40)	
rs3176123	2.9 kb 3' (in 3'UTR)	AA	396 (69)	108 (76)	0.22
		AC	164 (28)	31 (22)	
		CC	17 (3)	3 (2)	
		A	956 (83)	247 (87)	0.09
		C	198 (17)	37 (13)	
rs1962	3.8 kb 3' (in 3'UTR)	CC	35 (6)	9 (5)	0.82
		CT	202 (34)	51 (34)	
		TT	355 (60)	92 (61)	
		C	272 (23)	69 (23)	0.92
		T	912 (77)	235 (77)	
rs1042579	exon (A473V)	CC	299 (79)	118 (78)	0.43
		CT	73 (19)	33 (22)	
		TT	8 (2)	1 (1)	
		C	671 (88)	269 (88)	0.93
		T	89 (12)	35 (12)	

Supplementary Table 1. Genotype and allele frequencies of common SNPs in *THBD* gene in patients with aHUS and in healthy subjects**.** SNPs in the *THBD* gene were selected, based on the HAPMAP, using the SNP tagged approach (SNPs with frequency > 0.05 and $R^2 \geq 0.8$) and genotyped using the Sequenom Massarray technology. P-values were calculated using standard chi squared testing, with 1 and 2 degrees of freedom for alleles and genotypes, respectively. Genotype frequencies of SNPs in the controls and cases followed Hardy-Weinberg equilibrium. We also found rare flanking SNPs, 2 controls with G->C at base position -57, 1 control with C->T at base position -11, 2 controls with G->T at base position 2049, and 6 controls and 3 patients with A->G at base position 2079. All of these were heterozygous.

Supplementary Appendix 1

Case Reports

F635, pedigree #185. The proband is a 24 year-old man who had four episodes of HUS between 1 and 3 years of age, that led to end stage renal disease. The patient is now stable and on chronic hemodialysis. None of the episodes was preceded with a diarrhea prodrome. He had five brothers and three sisters. Three of the brothers (including twins) had HUS in early childhood and died during acute episodes of the disease. A younger sister, now 15 years old, also had an episode of HUS during infancy, but she recovered renal function, although she has persistent proteinuria. None of the proband's affected siblings had diarrhea prodromes. There are two older brothers and two older sisters who have never had HUS and they are alive and well. There is no known consanguinity between the parents, who are also healthy.

The proband was screened for complement genes (*CFH, MCP, CFI, C3* and *CFB*) and no mutations were found. Serum C3 was slightly reduced (82 mg/dl, normal values 90-180 mg/dl) and C4 concentration (30 mg/dl, normal values 10-40 mg/dl) was normal. Serum CFH levels were normal (515.6 mg/l, normal values 350-750 mg/l).

F163, pedigree #008. The proband is an 8 year-old boy and is the youngest of three brothers. The oldest died at the age of 8 months of HUS. The second sibling is a 10 year old boy. At present, this brother and the parents are healthy. The proband had three episodes of HUS, between 6 months and 2 years of age. All episodes were triggered by upper respiratory tract infections and characterized by acute renal failure, hypertension, hematuria and proteinuria. The boy was treated with plasmapheresis, blood transfusions and hemodialysis, always obtaining complete remissions. The proband, his healthy brother and the parents were screened for complement genes (*CFH, MCP, CFI, C3* and *CFB*) and no mutations were found. Serum C3 concentration was normal on one occasion (126 mg/dl) and slightly reduced (85 mg/dl) on another. Serum C4 concentrations were normal on both occasions (30 and 28 mg/dl). CFH levels (492 mg/l) were also normal. The activity of the vWF-cleaving protease, ADAMTS13, was normal (activity 110%, normal values 50-150%).

S884. The proband is a 4 year-old boy who developed HUS at the age of 15 months, after a febrile viral illness. The episode was characterized by anemia, thrombocytopenia, increased LDH, with mildly increased serum creatinine (0.6 mg/dl) and proteinuria. Serum C3 (68 mg/dl) was low, while C4 (11 mg/dl) was in the normal range. The boy was treated with plasma infusions and blood transfusions, obtaining a complete remission. He was screened for complement genes (*CFH, MCP, CFI, C3* and *CFB*). On our initial screen, an apparent heterozygous mutation in exon 23 of CFH (C→T at base 3572 causing a S1191L mutation) was detected. However, additional sequencing with different primers confirmed that no mutation existed in CFH. The original error was due to regions of high homology between CFH and CFHL genes. CFH levels (417 mg/L) were normal. No mutations were found in MCP and

CFI. ADAMTS13 activity was normal (81%). There is no relevant family history. The parents are from Yemen.

S511. The proband is a 10 year-old girl who developed HUS at the age of 3 years. She had two consecutive episodes of HUS with severe anemia and thrombocytopenia, and renal impairment (serum creatinine 0.7-1.4 mg/dl), both associated with low C3 levels (40-47 mg/dl). She responded well to plasmapheresis recovering normal renal function, but with persistant mild hypertension. Neither episode was associated with diarrhea or pneumococcal infections. The girl was screened for complement genes (*CFH, MCP, CFI, C3* and *CFB*) and no mutations were found. There is no relevant clinical history in the family.

S015. The proband is a 15 year-old boy who presented with fulminant HUS at the age of 6 months, becoming dialysis-dependent. At the age of 6 years he received a cadaveric kidney transplant, but HUS recurred in the allograft three days after the transplant, with loss of the kidney. Since that time the patient remains on dialysis. Serum C3 levels were normal (92 mg/dl), whereas C4 levels were slightly increased (49 mg/dl). CFH levels (418 mg/l) and ADAMTS13 activity (99%) were normal. The mother died a few years ago due to complications of idiopathic pulmonary fibrosis associated with fulminant pulmonary hypertension. The proband and his healthy father were screened for complement genes (*CFH, MCP, CFI, C3* and *CFB*) and no mutations were found.

S665. The proband is a 23 year old man from Italy who had an episode of HUS at the age of 6 years. He was treated with plasma exchange and recovered. Since then, he has no further episodes. At the last follow-up in 2004, his renal function was borderline normal (serum creatinine 1.24 mg/dl). The patient was screened for *CFH, MCP, CFI, C3* and *CFB*, and no mutations were found. Plasma ADAMTS13 activity are normal (78%). C3 (94 mg/dl), C4 (14 mg/dl) and CFH (515 mg/l) levels are normal.

S924. The patient is a 19 year old woman from the USA, who developed aHUS at the age of 15 years, a few days after receiving a cadaveric kidney transplant. She had severe microangiopathic hemolytic anemia and thrombocytopenia. A biopsy of the graft revealed typical features of thrombotic microangiopathy. Graft function rapidly deteriorated and she developed chronic renal failure. The patient was adopted. The adoptive parents did not have any information on the cause of the primary kidney disease, or whether there was a family history of aHUS. The patient was screened for *CFH, MCP, CFI, C3* and *CFB*, and no mutations were detected. C3 (106 mg/dl) and C4 (30 mg/dl) levels are normal. CFH (949 mg/l) levels are slightly elevated.

Supplementary Appendix 2

International Registry of Recurrent and Familial HUS/ TTP

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